Roles of Novel Protein Bga1 in Fission Yeast Cytokinesis

By

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Abstract:

Cytokinesis is an intricate part of the cell cycle in which an actomyosin ring assembles in the medial division plane, constricts, and then is disassembled. This is followed by extracellular matrix (ECM) deposition/remodeling and cell separation. These steps are conserved from yeast to mammals. We use the fission yeast *S. pombe* as the model organism to study cytokinesis. The *S. pombe* septum (an ECM) is a trilaminar structure assembled during ring constriction and consists of the primary septum flanked on either side by the secondary septum. The primary septum is digested at the end of cytokinesis to yield two new cells. Correct septum formation and digestion is vital for cell proliferation and survival. Septa are predominantly composed of polysaccharides that are synthesized by conserved glucan synthases. Because of this, glucan synthases are common targets for anti-fungal drugs. We have identified an essential gene, Bga1, which is associated with β-glucan synthase Bgs1 during cytokinesis. We hypothesize that Bga1 operates with Bgs1 and other cytokinesis genes to organize septum formation during cytokinesis. A yeast-2-hybrid screen was performed using Bga1 as bait to look for new binding partners. Rtn1 was a candidate gene isolated in the screen and has been implicated in cell wall synthesis

Introduction:

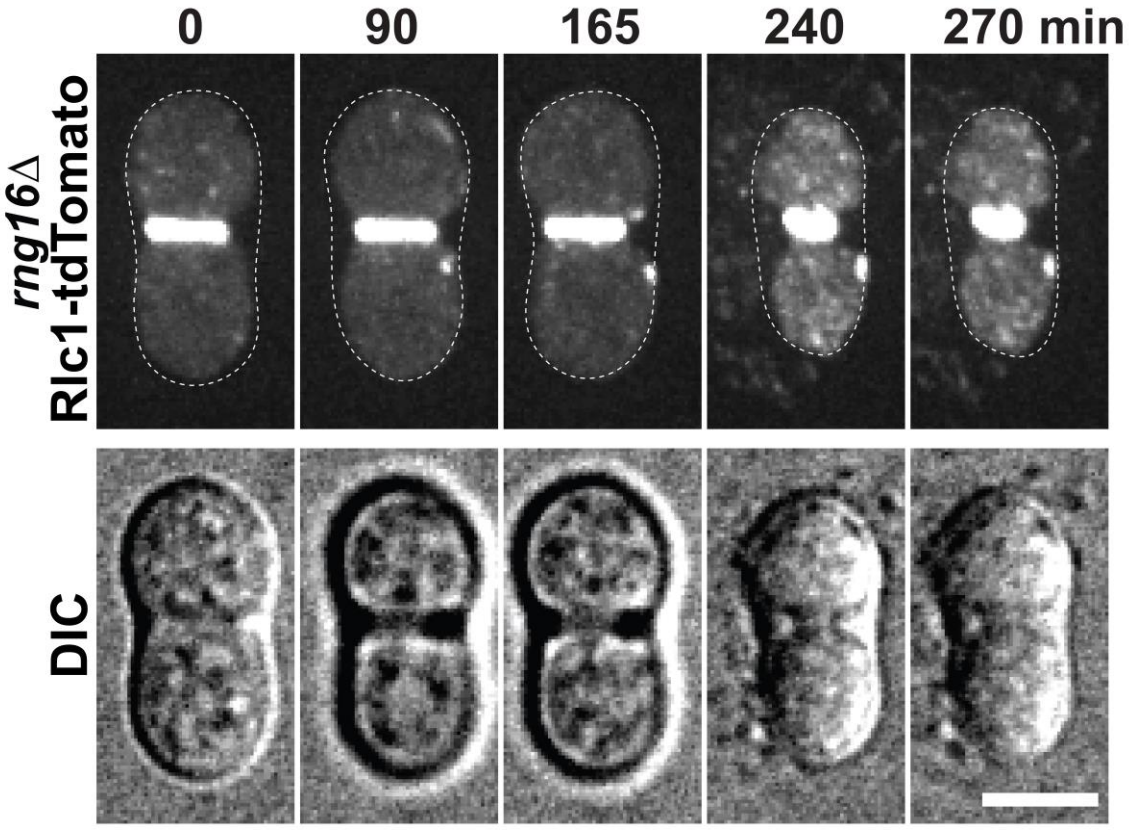
Cytokinesis is highly conserved from animal to fungal cells and is an integral process for cell integrity. It is also the final step of the eukaryotic cell cycle in which, following the completion of mitosis, the cleavage furrow ingresses and allows for the separation and formation of two new and identical cells. For animal cells, the formation, maintenance, and closure of a contractile actomyosin ring (CAR), which is tied to the accumulation of plasma membrane material, is necessary for furrow formation. Fungal cells are different because they are enveloped by a cell wall which regulates osmotic pressure. The functional analog for the cell wall in fungal cells is the extracellular matrix found in animal cells. Animal cells are encompassed by a structure made of proteins and polysaccharides generally referred to as the extracellular matrix. This matrix does not provide the osmotic support the cell wall does, but the function is still similar, and some of the extracellular matrix polymers have been portrayed as being crucial for cytokinesis. The presence of a cell wall necessitates that the CAR contracts in coordination with the biosynthesis of a unique cell wall structure called the division septum. The septum is tri-laminar structure composed of a middle primary septum (PS) flanked on either side by a secondary septum (SS). The final step of cytokinesis is the separation of cells via cell wall and PS degradation. The proper septum formation and subsequent degradation are imperative processes for the integrity and survival of the cell[5].

In *Schizosaccharomyces pombe* there are a few main glucose polysaccharides that are the structural polymers of the cell wall. Branched β(1,6)glucan is found in the cell wall as well as the SS; major branched β(1,3)glucan and α(1,3)glucan are located in the cell wall and both the PS and SS; and lastly the minor linear β(1,3)glucan is located mainly in the PS and minimal amounts in the cell wall.

To manufacture these structures, *S. pombe* has four essential glucan synthases. All of these localize to the septum, to the CAR, and to growing poles. Bgs1 (β-glucan synthase 1) and Ags1 (α-glucan synthase 1) simultaneously show up before septum synthesis at the division site, but Bgs4 (β-glucan synthase 4) does not localize until after septum initiation. Bgs1 is in charge of β(1,3)glucan and PS synthesis, while Ags1 is responsible for α(1,3)glucan and SS synthesis. Bgs1 and Ags1 are both vital for cell integrity during late mitosis and even during polarized growth, whereas Bgs4 functions in mostly cell wall synthesis[1].

Schizosaccharomyces pombe is a fission yeast strain used by our lab as a model system to study the cell cycle, and in particular the later steps in mitosis. Cytokinesis in *S. pombe* resembles mammalian cytokinesis. They both have the same four conserved steps: selection of the division plane, assembly of the contractile ring, constriction and disassembly of the contractile ring, and the separation of daughter cells by plasma membrane fusion. In addition, *S. pombe* has a quick generation time and is genetically tractable which makes it perfect as a model organism[5].

This purpose of this research is to investigate the role of a novel protein, Bga1, as it functions in cell division and cytokinesis in the model organism (*S. pombe*). Using Tetrad Fluorescence Microscopy, we found that *bga1Δ* cells died from cytokinesis failure. The constriction of the actomyosin ring was significantly delayed in mutants when compared to wild type cells (Figure 1). Most cells eventually lysed due to failed/defective septum formation. Electron microscopy of *bga1* mutants showed a thin and uneven primary septum and thickened secondary septum and cell wall (Figure 2).



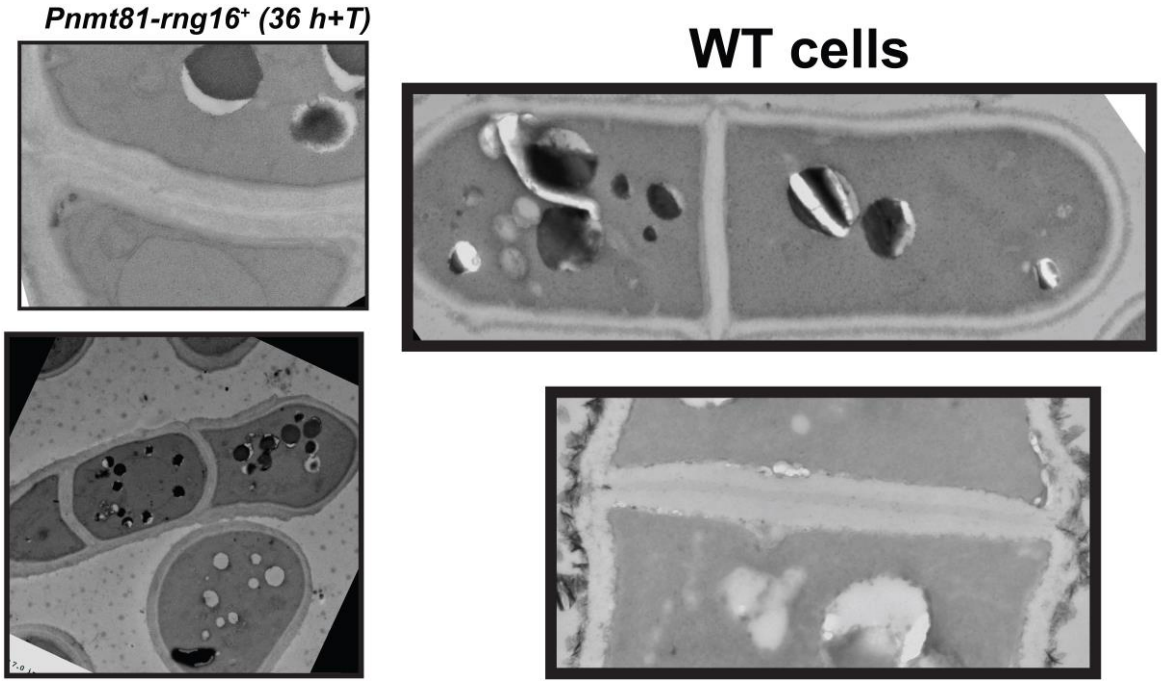


Figure 1(top): shows the delay in contraction by the contractile ring during cytokinesis.
Figure 2(below): Displays the repression phenotype of Bga1(Rng16) as compared to wt cells. Of particular importance is the enlarged secondary septum and improperly formed primary septum.

My thesis project is divided into two parts where I attempt to study the function of Bga1 in cytokinesis.

Aim1- To generate a temperature sensitive mutant for Bga1- We used the marker reconstitution technique to identify new temperature sensitive mutants for Bga1.

Aim2- To identify novel binding partners for Bga1- We used the Yeast two-hybrid screen to identify novel binding partners for Bga1.

Research Aim 1

To generate a temperature sensitive mutant strain for Bga1.

Introduction:

Bga1 is an essential gene, studying its function and genetic interactions with other genes has been complicated. Currently we have been studying Bga1 function by modulating its expression, however repressing a protein takes long time durations and it is hard to control the amount of protein in the cell. A fast acting temperature sensitive mutant would allow easier control of protein levels and would allow us to better ascertain the function of the protein. To create this mutant we used the technique described by Tang, X., et al[3].

Methods:

To create a fast acting temperature sensitive (ts) mutant of Bga1 we carried out marker reconstitution mutagenesis (Figure 3).

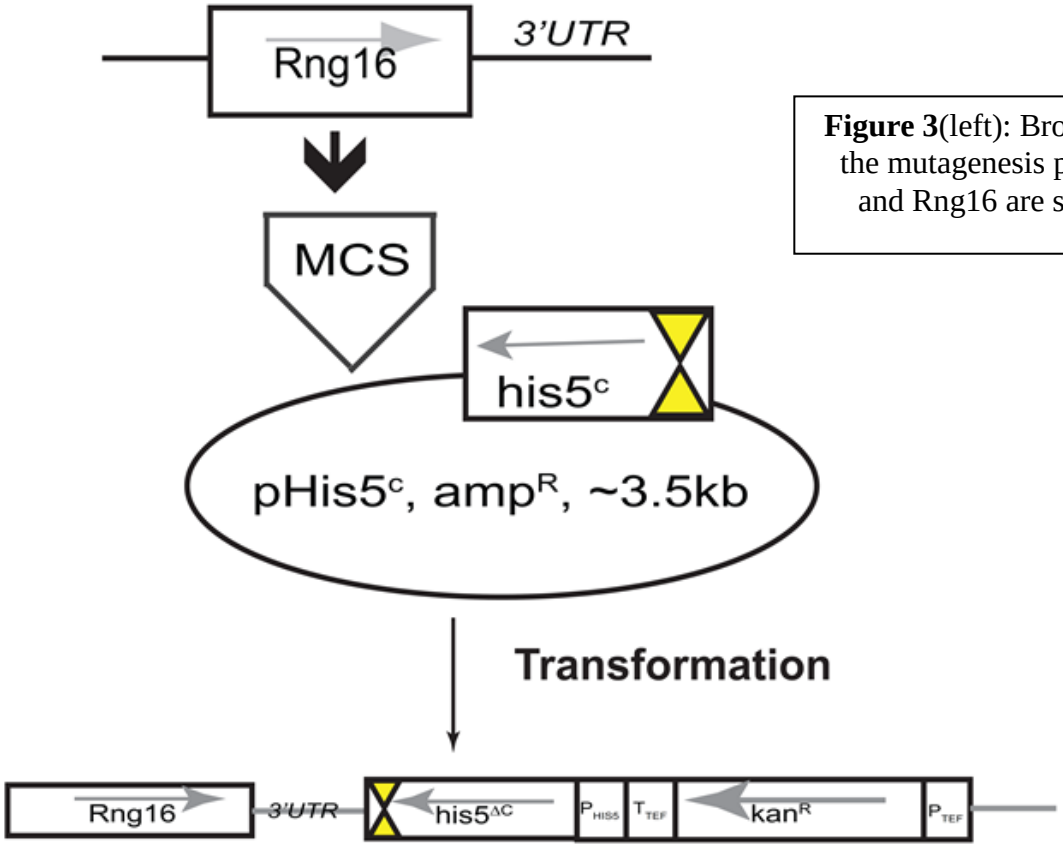


Figure 3(left): Broad overview of the mutagenesis process. (Bga1 and Rng16 are synonymous)

This process was carried out in 3 steps:

Part1: Cloning his5$^{\Delta c}$ downstream of Bga1 gene (Figure 3, top)

We determined the 3'UTR of the novel gene, Bga1, and then added the reverse complement of the end of his5$^{\Delta C}$ sequence at its end. A PCR reaction using primers upstream and downstream of the end of the 5' UTR, Hifi^{+} polymerase, and the his5$^{\Delta C}$-kan^{R} plasmid (JQW591) as a template was then performed. The PCR product was then transformed with the strain JW4821-1 *h- ade6-M210 his5Δ leu1-32 ura4* and kan^{R} colonies were selected for.

Part2: Cloning Bga1 into plasmid with his5^{c} (Figure 3, plasmid)

The cloning of Bga1 into pHis5^{C} took place with a forward primer (F1) which included a restriction site upstream of its start codon, and also a restriction site in the reverse primer (R1). iProof PCR using the F1 and R1primers as well as genomic DNA as a template was performed, and then TOPO cloning was used to amplify this PCR product as well as make cuts in both the product and pHis5^{C}. The new plasmid containing *bga1*$^{+}$*-his5*^{C} was then transformed and ligated with *E. coli.* This new *bga1*$^{+}$*-his5*^{C} plasmid was then used as a template for mutagenic PCR. This cloning is depicted in the figure shown[3].

Part 3: Mutagenic PCR

Error-Prone mutagenic PCR was then utilized to produce random mutations in the new plasmid containing *bga1*$^{+}$*-his5*^{C}. A mutagenic cocktail was created to include with the normal PCR reagents to promote mutations. This mutagenic cocktail, along with primer WU1464 His5-2F (P0 from Tang et al.; starts at +364/651bp in his5 gene sequence) with Forward primer (F1), is added to the PCR reagents.

Mutagenic cocktail concentration	1%	2%	4%	6%	8%
Mutants picked	23	27	19	8	10

Chart 1(above): Shows the relative number of mutants found after transformation with varying amounts of mutagenic cocktail.
Chart 2(below): The ingredients for the mutagenic cocktail are shown.

Reagent	[stock]	Volume	[final]
dTTP	100 mM (supply center)	80 µl	8 mM
dCTP	100 mM (supply center)	80 µl	8 mM
$MgCl_2 \bullet 6H_2O$	141 mM	340 µl	48 mM
$MnCl_2$	10 mM (PMP kit)	500 µl	5 mM

A special PCR program (EP-PCR) is used which only runs for 15 cycles instead of the normal 25-30 cycles, further promoting random mutations in the *bga1*$^{+}$*-his5*^{C} plasmid. This PCR product was then purified and used to transform the kan^{R} strain generated in Part I and plate on YE5S. After 48 hours these were replica plated to EMM–his plates, and then after about 8 days, these were replica plated to EMM–his and YE5S+PB plates consecutively, and grew the YE5S+PB plates at varying temperatures (19, 25, 30, 32, and 36°C) overnight. Colonies that were dark on PB at higher temperatures but not lower ones were picked from the EMM–his plates and re-streaked to EMM–his again, and kept for further characterization.

Results:

The goal of the mutagenesis was to obtain a *ts mutant* with a mutation in the open reading frame (ORF) of the novel gene Bga1. Through many rounds of marker reconstitution mutagenesis many excellent candidates were found to be dark red on the YE5S+PB plates at 36°C, but remained light at 25°C. The colonies were grouped according to the percentage of mutagenic cocktail included in the PCR so the best ratio could be determined. The promising colonies were then screened on a microscope to check that their phenotype at 36°C matched that of *bga1Δ*, and then if the phenotype of the colony matched that of the wild type. Those colonies that then fit these criteria had their Bga1 sequence extracted and amplified before it was sent for sequencing. All of the candidates did not possess a mutation in the Bga1 ORF, but instead most had a mutation in either the 3' or 5' UTRs. None of these were usable for our purposes because the mutation was not in the ORF. It is believed that obtaining a functional mutant was difficult due to a few reasons. One of which is the fact that Bga1 is a small gene with only 181 amino acids. The other is that Bga1 is an essential gene, so any mutation in the ORF could be detrimental make the strain inviable, and therefore incapable of being temperature sensitive.

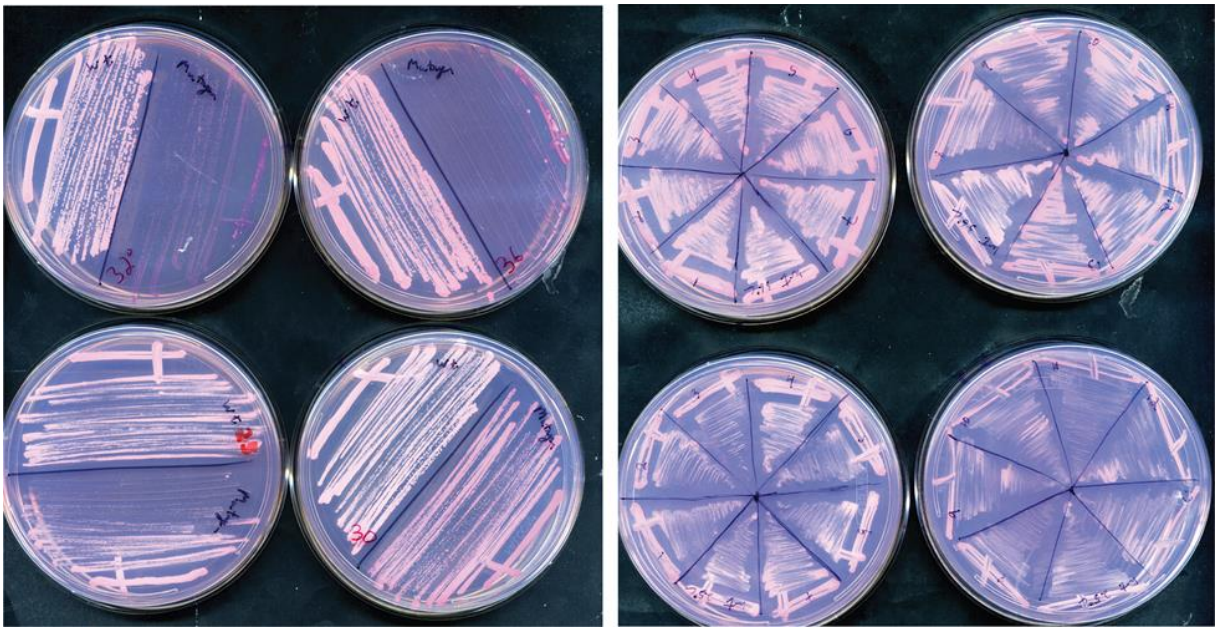


Figure 4(above): The streaking of different candidates onto YE5S+PB plates is shown at varying temperatures. The darker the streaks the more dead cells there are present.

Research Aim 2:

To discover novel binding partners for Bga1

Introduction:

The role of Bga1 in cytokinesis is not well understood other than that it is an essential gene and that it interacts with Bgs1. Uncovering more binding partners would allow for the function of this novel gene to become more clear, as well as the function of Bgs1 and Bga1 in cytokinesis.

Materials and Methods:

Reagents required- Bga1 'bait' was cloned into the GBT9 plasmid and *S. pombe* library in pACT2 was used as 'prey'. These plasmids contained the leucine and tryptophan selection markers. Positive interaction between the bait and prey resulted in expression of the uracil and histidine selection marker. Leaky expression from the histidine marker was controlled by adding 50 mM of 3-aminotriazole 3-AT to the medium.

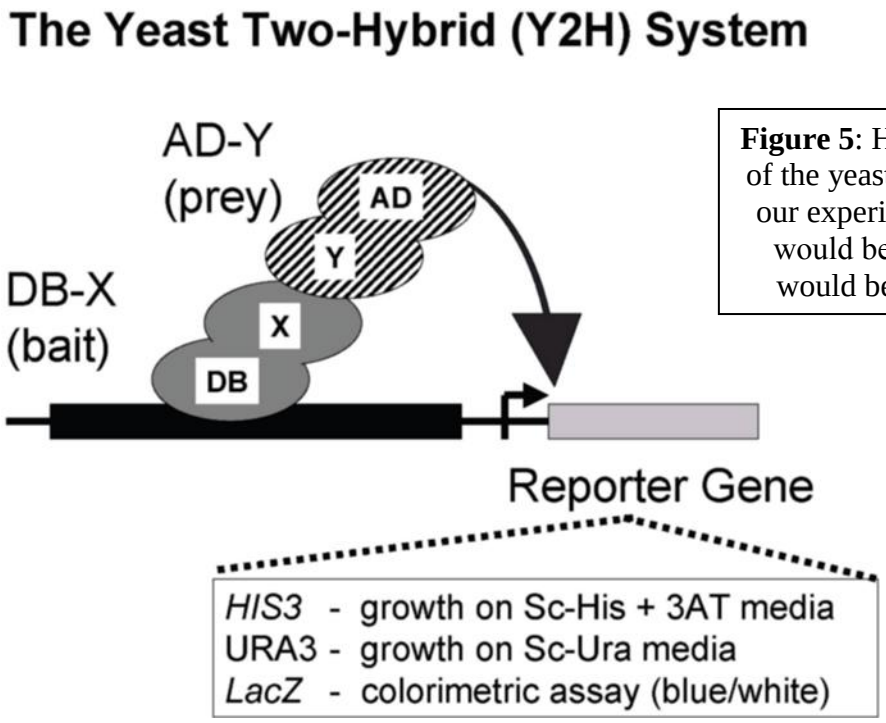


Figure 5: Here is a schematic view of the yeast-two-hybrid system. In our experiments, the Bga protein would be the "X" and the "Y" would be any candidate gene.

Method- *Saccharomyces cerevisiae* was grown and diluted in YPD media for three days prior to transformation in order to generate the required volume of cells for a successful transformation. The cells were plated onto SD-L-W-U-His+ 3-AT quadruple selection plates and incubated at 30°C until colonies started to form. An X-gal overlay assay was used to confirm in the colonies that grew on the drop-out plates.

Positive colonies were then picked and screened via colony PCR. Primers specific for the library plasmid (pACT2) were used to check for an insert and the size of the insert. Several candidates were confirmed at this point, and their plasmids were isolated and sent for sequencing to check for genes of interest.

The corresponding pACT2 plasmid was isolated and then a confirmatory yeast-two-hybrid screen was performed using these plasmids to confirm the interaction. X-gal overlay assay was used to confirm the protein to protein interactions.

Aim 2 Results:

Due to the ongoing nature of the research, only preliminary data can be displayed. One gene, which was confirmed to interact with Bga1 in the yeast-two-hybrid assay, was found to be Rtn1.

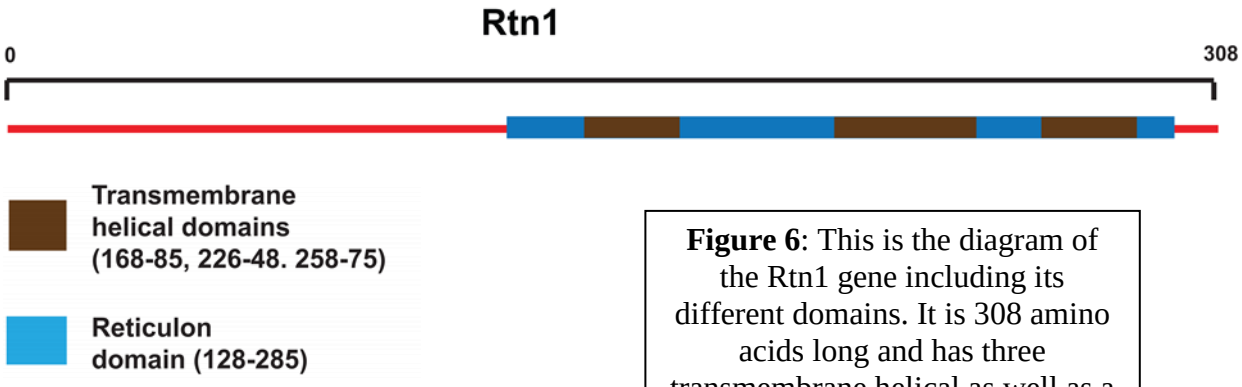


Figure 6: This is the diagram of the Rtn1 gene including its different domains. It is 308 amino acids long and has three transmembrane helical as well as a reticulon domain at its c-terminus.

In literature, Rtn1 is predicted to be involved in ER network membrane organization. Additionally, it was found that Rtn1 overexpression leads to lessor cell wall glucan content as well as cell separation defects during cytokinesis[7]. It has three transmembrane helical domains and also a reticulon domain in its 308 AA sequence. There was no phenotype for *rtn1*Δ reported in literature, but upon waking up this strain from the lab's strain library, it was found that *rtn1*Δ has no phenotype at 25°C, but there was an obvious phenotype at 36°C as is shown in the figure below. The cells were substantially longer in the *rtn1*Δ strain. By further studying the binding partner of Bga1, the function of Bga1 will be better understood.

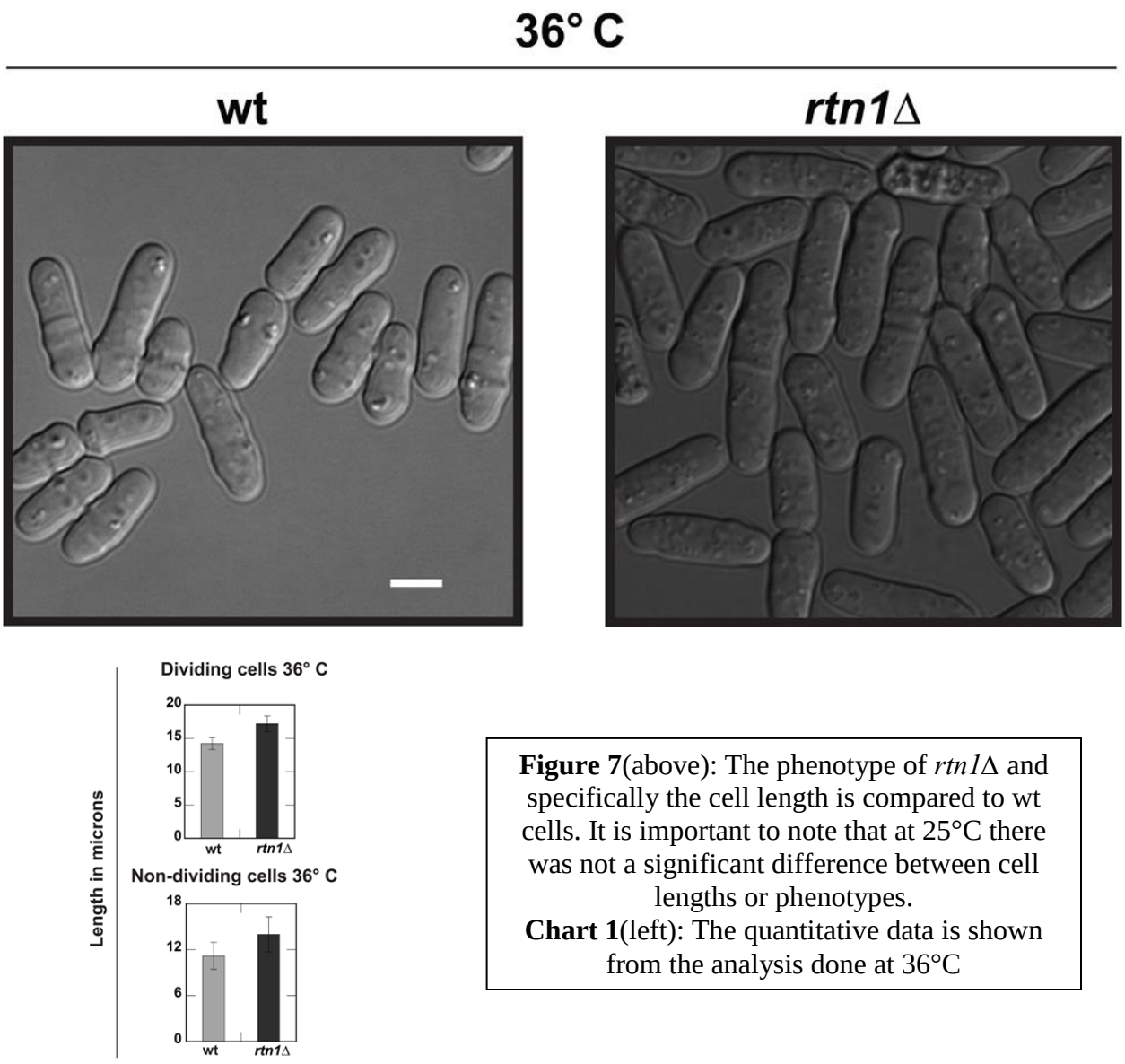


Figure 7(above): The phenotype of *rtn1*Δ and specifically the cell length is compared to wt cells. It is important to note that at 25°C there was not a significant difference between cell lengths or phenotypes.
Chart 1(left): The quantitative data is shown from the analysis done at 36°C

Discussion:

The results from the first part of my research confirmed a suspicion I had from the start: because Bga1 is a small and essential gene, it would be extremely difficult to obtain a workable mutant. After a round of unsuccessful mutants, I decided to track how much mutagenic cocktail was included in the mutagenic PCR reactions. Previous attempts to mutate genes in the lab had taken place with varying success and there had been no agreement as to what the correct percentage of cocktail added should be. I believed varying this would give me a good indication of which direction to go, and also to guide future research. Additionally, further reading has revealed that perhaps specifically altering the concentration of magnesium ions in the cocktail would further alter the results. This is due to the stabilizing effect magnesium ions have with TAQ-Polymerase[6].

Another issue I encountered was making the decision of how dark is dark enough when deciphering colonies on the YE5S+PB plates. These pink plates were helpful, but choosing amongst many shades of dark pink was arduous and sometimes not easily objective. For the most part, it is believed that the lack of a working mutant is due mainly to the fact that the gene is small and essential, so any mutation was probably very harmful to the organism.

The road to garnering a successful binding partner with Bga1 was not as unforgiving as obtaining a mutant. In the very first yeast-two-hybrid screen, 42% of the sequenced candidates were Rtn1. From there, the interaction was later confirmed via the protocol, but we decided to try and find other binding partners in subsequent yeast-two-hybrid screen. This was due in part because Rtn1 is not an essential gene, and also because Bga1 showed a lot of promise in unveiling more of the regulation of cytokinesis. There were many false positives found through the screen that were confirmed to be false via the beta-Gal assay.

Rtn1 has been evaluated before in *S. pombe* by Carlos Godoy's lab under the alias of Cwl1. Their research gathered that the overexpression of Rtn1 causes cell lysis in the absence of an osmotic stabilizer, but that the disruption of the gene caused no noticeable phenotype[7]. The *rtn1*Δ phenotype was again explored by Johan-Owen De Craene and Jeff Coleman in regards to an altered ER morphology in *S. cerevisiae*. They found that the ER in *rtn1*Δ cells is predominately cisternal rather than reticular, though there was no change in ER surface area. Another discovery this study made was that most Rtn proteins not only found in a wide range of eukaryotes, but are defined by two membrane-spanning domains flanking a conserved hydrophilic loop. Additionally, they were able to determine that the hydrophilic loop of Rtn1p binds to a subunit of Sec6p, and the overexpression of the loop results in accumulation of secretory vesicles (De Craene JO 2006). It would follow that in *S. pombe* Rtn1 has a similar function as a cortical ER protein, and in organizing the structure of the ER, such as maintenance of tubular ER morphology. Also, it could potentially play a direct or indirect role in vesicle transport of glucan synthases[8]. This would explain the interaction with Bga1 and the subsequent lethal phenotype when overexpressed in cells. The overexpression phenotype is the lessoning of cell wall content and eventual lysis, so it would appear as if the cell wall was either missing a component or was not being created in the first place.

Acknowledgments:

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Strain table:

Name	Alias	Detailed Genotype Description	Source	Date	Comments
JW81		*h- ade6-210 ura4-D18 leu1-32*		3/11/98	
JW4821-1		*h⁻ his5Δ ade6-M210 leu1-32 ura4*	JW4620-2 x JW729	08/24/12	VC, *h⁻* version of JW4710
JW6173-1,2,3		*h⁻ bga1-his5ΔC-kanMX6 his5Δ ade6-M210 leu1-32 ura4*	JW4821	06/04/14	RD; *his5ΔC-kanMX6 downstream of bga1 3'UTR*
JW6877-1,2,3,4		*h⁻ kanMX6-P81nmt1-bga1 ade6-210 leu1-32 ura4-D18*	JW81	5/4/15	RD; no phenotype even though PCR correct
JW6166-1,2,3,4		*h⁻ kanMX6-P3nmt1-mEGFP-bga1 ade6-210 leu1-32 ura4-D18*	JW81	05/28/14	RD
JW6167-1,2,3,4		*h⁻ kanMX6-P41nmt1-mEGFP-bga1 ade6-210 leu1-32 ura4-D18*	JW81	05/28/14	RD
JW6787-1,2,3,4,5		*kanMX6-Pbga1-mEGFP-bga1 bgs1Δ::ura4⁺ Pbgs1⁺-tdTomato-bgs1⁺:leu1⁺ade6-M210? his3-D1? leu1-32 ura4-D18*	JW6773-1 x JW6717	06/04/14	RD; -1,-2 is h^-; -3 and -5 are h^+ ade6-M210 marker not checked

Plasmids used:

JQW591	pH5Dc-KanR	P_{TEF}-kanMX?-T_{TEF}-P_{his5}-his5□c (missing 77bp), amp^R	P	Mohan	06/08/12	VC, Tang et al. Yeast, 2011
JQW797-1,2,3,4,5,6		pACT2 pombe cDNA library,ampR	B	JQW798	2/25/15	YZ, automated converted from lambdaACT cDNA library (ATCC: 87289) for yeast-two-hybrid screening
JQW270		pGBT9 (contain Gal4-DNA-binding domain), AmpR, TRP1	B		11/15/09	Gift from Burghes lab